Isometric Primer Extension Method and Kit For Detection and Quantification of Polynucleotides

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 09/862,417, filed May 23, 2001, which claims benefit of priority to U.S. Provisional Patent Application No. 60/209,987, filed June 8, 2000, all of which are incorporated by reference herein in their entirety.

BACKGROUND

1. Technical Field

The present invention is generally related to methods, compositions, and kits for detecting and/or quantifying polynucleotides.

2. Related Art

Conventional methods for detecting and quantifying specific sequences of nucleic acids such as DNA and RNA include southern blotting, northern analysis, and RNase protection assays, and Polymerase Chain Reaction (PCR), among other methods. However, if the detection of a specific RNA species in a sample is considered, Northern blotting and RNase protection assay present limitations in efficiency, labor intensiveness, accuracy, high cost, sensitivity, greater RNA sample requirement, specialized equipment, and a large amount of bio-hazardous and radioisotopic waste material that are generated. In particular, both Northern blotting and RNase protection assays require 2-3 days for completion of the analyses. In addition, Northern blotting requires running an RNA gel, transferring the RNA to a solid support, preparing a probe, and carrying out a hybridization reaction. About a 5 μg sample is required for for adequate sensitivity. Northern blot analysis is based on the principle of hybridization between the target and the probe nucleic acid. Moreover, the cost per reaction is fairly high, as is the amount of biohazardous and radioisotopic waste material that are generated.

Similarly, an RNase protection assay requires 2-3 days to obtain appropriate results. The experimental procedure requires preparing template DNA, preparing RNA probe, carrying out hybridization reactions, enzyme digestion reactions, and running a gel. To obtain good results, at

least a 1 μg sample of the target RNA is required. The principle behind RNase protection assay is a combination of hybridization and enzyme digestion reactions. As in the Northern blotting method, it is expensive to carry out this reaction. Furthermore, biohazardous and radioisotopic waste products are generated in high amounts.

U.S. Pat. No. 5,846,710 discloses using a primer extension technique to screen for variant DNA molecules. However, this patent does not disclose detecting a target DNA or RNA in a sample.

U.S. Pat. No. 5,994,079 discloses forming an RNA/DNA hybrid by annealing a DNA primer to a specific RNA and extending the primer by using reverse transcriptase. The hybrid is detected by an antibody specific for the RNA/DNA hybrid. However, this patent does not disclose detecting a target DNA or RNA in a sample as in the present invention.

It is recognized that there is a need in the art for a nucleic acid detection method that is simple, costs less time, is sensitive, cost effective and has a low adverse environmental impact.

SUMMARY

Aspects of the present disclosure generally provide methods, compositions, and kits for the detection, quantification, or identification of a nucleic acid of interest or a target polynucleotide. The nucleic acid of interest or the target polynucleotide is known nucleic acid or polynucleotide having a known nucleotide sequence. One aspect provides a method for detecting or quantifying a known target polynucleotide including producing equal length extension products using a first primer that hybridizes to the target polynucleotide, hybridizing the equal length extension products to a second primer and producing extension products from the second primer, and detecting the extension products from the second primer. The signal detected from the extension products formed from the second primer can be correlated to the amount of target polynucleotide in a sample.

Another aspect provides a method for detecting a target polynucleotide by hybridizing a first primer to the target polynucleotide and forming equal length primer extension products using a reaction mixture having nucleotides consisting of X, Y, and Z, wherein X and Y are different purine non-terminator nucleotides, and Z is a pyrimidine non-terminator nucleotide; or X and Y are different pyrmidine non-terminator nucleotides, and Z is a purine non-terminator

nucleotide. The extension products are hybridized to a second primer which can be immobilized on a solid support. The second primer is extended with at least one nucleotide having a detectable marker using a portion of the equal length extension products as a template. The amount of detectable marker can then be correlated with the amount of target polynucleotide.

Yet another aspect provides a kit for quantification of a target nucleic acid. The kit includes a first primer complementary to a known polynucleotide sequence of the target nucleic acid, and a second primer complementary to an extension product formed from the first primer. The second primer is not complementary to the first primer. One or more enzymes for performing a primer extension reaction are also included. The kit includes a non-terminator nucleotide mixture formulated to produce equal length primer extension products. One exemplary nucleotide mixture is provided having nucleotides consisting of X, Y, and Z, wherein X and Y are different purine non-terminator nucleotides, and Z is a pyrimidine non-terminator nucleotide; or X and Y are different pyrmidine non-terminator nucleotides, and Z is a purine non-terminator nucleotide. The nucleotides can be labeled with a detectable marker. The detectable marker may comprise an enzyme or protein moiety, radioactive isotope, a fluorescent moiety, or a chemical group such as biotin. Moreover, the detecting or quantifying method step may be carried out by fluorospectroscopy or mass spectrometry.

Still another aspect of the disclosure provides a method for detecting or quantifying a target nucleic acid in a sample including: (a) preparing a primer or primers specifically matched to a predetermined position of the target nucleic acid; (b) annealing the primer or primers from (a) with the target nucleic acid under high stringency conditions to obtain a primer-nucleic acid duplex at the predetermined position of the target nucleic acid; (c) mixing the primer-nucleic acid duplex from (b) with a mixture including: (1) one or two or three types of free non-terminator nucleotides and at least one type of non-terminator nucleotide that is optionally labeled with a detectable marker, and (2) with or without a type of terminator nucleotide that is different from the one or two or three types of non-terminator nucleotides in (1); (d) performing the primer extension by enzymatic or chemical reaction in an appropriate buffer; and either (e) detecting or quantifying the amount of labeling signal on the primer extended nucleotides, or (f)

detecting or quantifying the amount of extended primers, for example using spectrometric techniques including but not limited to mass spectrometry.

The primer can be a nucleic acid primer, an oligodeoxyribonucleotide, an oligoribonucleotide, or a copolymer of deoxyribonucleic acid and ribonucleic acid. The nucleic acid of interest can be a deoxyribonucleic acid, a ribonucleic acid, or a copolymer of deoxyribonucleic acid and ribonucleic acid.

In an exemplary embodiment, the method may comprise using a mixture comprising a combination of non-terminator and terminator nucleotides as follows:

(a) dATP, dCTP, dGTP, ddTTP or ddUTP,

(b) dATP, dCTP, dTTP or dUTP, ddGTP,

(c) dATP, dGTP, dTTP or dUTP, ddCTP,

(d) dCTP, dGTP, dTTP, or dUTP, ddATP,

(e) dATP, dCTP, dGTP,

(f) dATP, dCTP, dTTP or dUTP,

(g) dATP, dGTP, dTTP or dUTP, or

(h) dCTP, dGTP, dTTP or dUTP.

Some of the enzymes used in the primer extension reaction of the invention include a template-dependent enzyme such as E. coli DNA polymerase I or the "Klenow fragment" thereof, T4 DNA polymerase, T7 DNA polymerase, T. aquaticus DNA polymerase, a retroviral reverse transcriptase, or a combination thereof.

These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of multiple primer extension reaction to detect and quantify a specific DNA sequence.

FIG. 2 is a schematic for using the multiple primer extension method to detect and quantify RNA.

FIG. 3 is a schematic of an exemplary multiple primer extension reaction to detect and quantify a specific polynucleotide sequence.

FIG. 4 is another embodiment of a multiple primer extension reaction to detect and quantify a specific polynucleotide sequence.

FIG. 5 is a line graph showing the absorbance at concentration of RNA versus absorbance at 405 nm. The data show that the detection of the primer extension products correlates with the amount of RNA in the sample.

FIG. 6 is a bar graph showing absorbance at concentration of RNA versus absorbance at 405 nm. The data show an exemplary diagnostic method of the present disclosure.

DETAILED DESCRIPTION OF THE VARIOUS EMBODIMENTS

Embodiments of the present disclosure are directed to methods, compositions, and kits for detecting and/or quantifying a known nucleotide sequence or a known polynucleotide using an isometric primer extension method. Isometric primer extension means producing equal length or substantially equal length polynucleotide extension products using enzymatic, chemical, or physical means and a template strand of a polynucleotide. Substantially equal length polynucleotide extension products means extensions products with a length that varies by less than about two nucleotides. One embodiment of the present disclosure provides a detection or quantification method including the steps of hybridizing at least one polynucleotide primer to a target polynucleotide having a known nucleotide sequence, for example DNA, RNA, or a combination thereof. Because the nucleotide sequence of the target polynucleotide is known or has been previously determined, the sequence of the primer can be designed to anneal to the single stranded target polynucleotide at a specific position of the target polynucleotide. The primer is then extended, for example enzymatically extended using a polymerase such as a DNA polymerase, reverse transcriptase, or a combination thereof. Conventional extension reactions extend primers by matching a complementary nucleotide to a specific nucleotide in a template polynucleotide strand.

It is well known that nucleotides include a nitrogen-containing base. This nitrogen-containing base can be a purine or pyrimidine base. Purine bases include adenosine ("A"), guanosine ("G"), hypoxanthine, and xanthine. Pyrimidine bases include thymine ("T"), cytosine ("C"), uracil ("U"), and orotic acid. It will be appreciated that the single letter abbreviations refer to the base or the nucleotide incorporating the specific base. Uracil is found only in RNA, and thymine is found in DNA. The standard or canonical Watson-Crick base pairs are A-U(T) and G-C. Thus, conventional polynucleotide extension reactions include nucleotide reaction mixtures having four different types of nucleotides each with a different nitrogen-containing base A, T, G, and C, to insure that each nucleotide in the template polynucleotide strand has its complementary base pair available in the extension reaction mixture. Without a complementary nucleotide in the reaction mixture to base pair with each nucleotide in the template strand, the extension reaction will stop at the nucleotide in the template strand that does not have its canonical base pair nucleotide present in the reaction mixture.

In one embodiment, the extension reaction mixture is formulated to prevent canonical base pairing with at least one nucleotide in the template polynucleotide. The extension reaction mixture includes a nucleotide mixture, for example a non-terminator nucleotide mixture. More particularly, the extension reaction mixture can be selected to include non-terminator nucleotides having two different purine bases in combination with nucleotides having the same pyrimidine base or nucleotides having at least two different pyrimidine bases in combination with nucleotides having the same purine base. Typically at least two nucleotides are added to the primer during the primer extension reaction, more typically about 3 nucleotides to about 20 nucleotides are added, even more typically at least 6 nucleotides are added to the primer. The primer itself is typically at least about 6 nucleotides in length but can vary in length from about 6 nucleotides to about 100 nucleotides, typically about 10 nucleotides to about 25 nucleotides, even more typically from about 15 to about 20 nucleotides in length.

In another embodiment, the non-terminator nucleotide mixture includes nucleotides consisting of X, Y, and Z, wherein X and Y are different purine non-terminator nucleotides, and Z is a pyrimidine non-terminator nucleotide; or X and Y are different pyrmidine non-terminator nucleotides, and Z is a purine non-terminator nucleotide. Exemplary non-terminator nucleotide

mixtures with nucleotides having at least two different pyrimidine bases and one purine base include: C and T; C and U; or T and U; in combination with A or G. Exemplary extension reaction mixtures with nucleotides having two different purine bases and one pyrimidine base include: A and G in combination with at least one nucleotide selected from C, T or U. Alternatively, the reaction mixture includes a terminator nucleotide such as a dideoxynucleotide.

In yet another embodiment, the target polynucleotide contains a known polynucleotide sequence or a known nucleotide in a specific position, and the extension reaction mixture can be formulated to omit one or more nucleotides complementary to a known nucleotide in the known target polynucleotide sequence. The specific target polynucleotide can then be detected and quantified by measuring the presence or absence of the signal generated by the label on the extended primer(s). As the extended primer is separated away from the free nucleotides, the extended primer is assayed for incorporation of the label.

Using the known nucleotide sequence of a target polynucleotide, the first primer is designed to hybridize to a specific portion of the target polynucleotide. A plurality of equal length (isometric) primers extension products can be made by formulating the primer extension reaction mixture to prevent canonical base paring with at least one nucleotide present in the template polynucleotide. Each primer will be extended until the polymerase reaches the nucleotide in the template strand for which there is no canonical complementary nucleotide in the extension reaction mixture. Thus, in general, each primer that attaches to each molecule of target polynucleotide will be extended with the same number of nucleotides, and the primer extension reaction will stop at the same place on each template polynucleotide. It will be appreciated that the target polynucleotide can be amplified, for example by PCR, prior to hybridization with the first primer.

The isometric primers can be accurately quantified and therefore used to quantify the amount of target polynucleotides by using labeled nucleotides in the primer extension reaction mixture. If there are many copies of the target polynucleotide, for example DNA, RNA, or combinations thereof in the sample, the number of copies of the primer extended product incorporating the labeled nucleotide will be correspondingly increased, contributing to a stronger overall signal. Thus, by comparing the strength of the signal observed in the unknown sample

with the signal obtained from a standardized amount of polynucleotide, it is possible to detect and/or quantify the amount of the target polynucleotide in a sample. In another embodiment, the specific target polynucleotide can be detected or quantified by measuring the amount of these equal length primer extended nucleic acid species using mass spectrometry.

Another embodiment provides a method using a plurality of primers having different nucleotide sequences, for example at least two primers. A first primer is designed to complementary hybridize to a specific sequence of a known target polynucleotide, for example under high stringency conditions. A second primer is designed to hybridize with the extension product resulting from the first primer (Figure 3). The 5' end of the first or second primer can be immobilized on a solid support. The solid support can be a pin, bead, glass, metal, plastic, chip, capillary, wafer, comb, array, or polymeric surface such as a multi-well plate. Additionally a linker molecule can be used to attach the primer to the solid support. Such linker molecules can include, for example, branched or unbranched alkyl chains ranging from C_1-C_{18}, a photocleavable moiety, amino acids, nucleotides, peptide nucleic acids and the like. The linker group can contain chemical or enzymatic cleavage sites for releasing the primer from the solid support.

Other methods for attaching the polynucleotide to the surface of a solid support are known in the art and include coating the surface with an immobilizing agent, for example an amino containing compound such as poly-lysine or an amino-silane such as 3-aminopropyltrimethoxysilane. Alternatively, the polynucleotide can be chemically modified or have a linking group attached that reacts with the surface of the solid support. The reaction between the polynucleotide and the surface on the solid support can be convalent, ionic, adsorptive, absorptive, magnetic, or a ligand:receptor interaction. For example, the polynucleotide can be modified to include an active silyl moiety to produce silanized polynucleotides. The silanized polynucleotides can be readily immobilized on glass surfaces.

An exemplary ligand:receptor interaction can be accomplished using biotinylated polynucleotides and a solid support modified with streptavidin. Ultraviolet light can be used to photoactivate binding of the modified polynucleotide to the surface or to release the polynucleotide from the surface.

After the first primer hybridizes to a target polynucleotide, it is extended, for example using DNA polymerase such as DNA polymerase I or reverse transcriptase or a combination thereof in the presence of a mixture of nucleotides. The nucleotide mixture includes non-terminator nucleotides having two different purine bases in combination with non-terminator nucleotides having the same pyrimidine base. Alternatively, the nucleotide mixture includes non-terminator nucleotides having at least two different pyrimidine bases in combination with non-terminator nucleotides having the same purine base. Thus, the nucleotide mixture is formulated so that the nucleotide mixture does not contain complementary nucleotides to at least one nucleotide present in the template strand.

The absence of this one type of free non-terminator nucleotide, for example the absence of one of A, T, U, G, or C from the reaction mixture causes the primer extension reaction to terminate where the missing nucleotide would have been inserted. Thus, an equal number of nucleotides are incorporated into each primer extension product because the primer extension reaction will stop at the same place on each template polynucleotide. As a result, each extension product from the first primer will have the same length (isometric primer extension). The extension product from the first primer is then captured by a second primer, for example an immobilized second primer. This capture is generally accomplished when the extension product hybridizes to the second primer via canonical Watson-Crick base pairing. It will be appreciated that the primer extension reaction products have at least two regions: (1) the primer region and (2) the extended region formed during the primer extension reaction. In one embodiment, the second primer is designed to complementary hybridize to the extended region of the extension reaction product from the first primer. The second primer hybridizes to a portion of the extended region of the first primer extension reaction product to form a double-stranded hybridization product, for example by canonical Watson-Crick complementary base pairing.

A portion of the first primer extension reaction product remains single stranded and serves as the template strand for a second primer extension reaction. The second primer is then extended using the first extension product as a template strand. In particular, the primer region of the extension product will typically serve as the template for the second primer extension reaction. The second primer is extended using a reaction mixture having a complete set of

nucleotides, for example A, T or U, G, and C, for continuous primer extension. Accordingly, the first primer can be designed to include at least four nucleotides each having different nitrogen-bases. At least one nucleotide in the nucleotide mixture for the second primer extension reaction is labeled with detectable marker. The second primer extension product is detected and or quantified by detecting or quantifying the signal from the one or more incorporated labeled nucleotides. Because all of the first primer extension reactions are of the same length, all of the second primer extension products will similarly be the same or about the same length because the extension reaction product of the first primer serves as the template strand for the second primer extension reaction. Moreover, each extension product from the second primer will incorporate the same number of labeled nucleotides, and therefore each extension product from the second primer will have an equal amount of detectable label or generate approximately an equal amount of detectable signal from the label. Quantification of these equally extended primers will quantify the number or amount of the target polynucleotide. If there are many copies of the target DNA or RNA in the sample, the number of copies of the primer extension products incorporating labeled nucleotides will be correspondingly increased, proportionally contributing to a stronger overall signal. Thus, by correlating the strength of the signal from extension reaction products produced using a template polynucleotide in a sample with the signal from extension products produced from a standardized known amount of DNA or RNA, it is possible to detect and/or quantify the amount of the target polynucleotide in the sample.

The length of the extension products of the second primer are limited by the length of the extension products of the first primer. The number of extension reaction products from the second primer can be quantified using a variety of known techniques based on size, including mass spectrometry, in particular time-of-flight mass spectroscopy. The amount of primer extension products can be correlated to the amount of target polynucleotide in a sample.

In another embodiment, the second primer is extended in the presence of four types of unlabelled nucleotides. After the extension, the extended polynucleotide can be stained by a dye or fluorescent dye such as cyber green, the concentration of the target polynucleotide can be measured by the amount of the signals from the dyes.

Still another embodiment of the present disclosure provides a kit for the detection or quantification of a single nucleotide or polynucleotide. An exemplary kit includes a first primer designed to hybridize to a target polynucleotide, preferably under high stringency. A polymerase such as DNA polymerase I, a reverse transcriptase, or a combination theref is also included to enzymatically extend the primers. The kit includes a second primer complementary to an extension product formed from the first primer. In one embodiment, the first and second primers are not complementary and cannot form a double-stranded complex. Instead, the second primer hybridizes to the extended region of the extension reaction product from the first primer.

The kit includes a non-terminator nucleotide mixture formulated to produce equal length primer extension products. The non-terminator nucleotide mixture generally includes non-terminator nucleotides having two different purine bases in combination with non-terminator nucleotides having the same pyrimidine base. Alternatively, the nucleotide mixture includes non-terminator nucleotides having at least two different pyrimidine bases in combination with non-terminator nucleotides having the same purine base. At least one of A, T, U, G, or C is omitted from the extension reaction so that at least one nucleotide of the template strand does not have its complementary non-terminator nucleotide in the reaction mixture. The non-terminator nucleotides can include a detectable label. The kit can also include a second primer immobilized on a solid support and buffer solutions or powders for making buffer solutions to conduct the extension reactions.

An exemplary non-terminator nucleotide mixture includes a combination of nucleotides consisting of X, Y, and Z, wherein X and Y are different purine non-terminator nucleotides, and Z is a pyrimidine non-terminator nucleotide; or X and Y are different pyrmidine non-terminator nucleotides, and Z is a purine non-terminator nucleotide.

Embodiments of the present disclosure are useful as diagnostic tools for the detection and/or quantification of gene expression in an organism, for example a primate. Such gene expression can be used to diagnose the organism with a pathology including but not limited to a genetic disorder, cancer, heart disease, predisposition for a disease or syndrome, viral infection, bacterial infection, HIV, hepatitis, diabetes, autoimmune disease, and the like. In some embodiments, the primers can be designed to hybridize with polynucleotides encoding

polypeptides known to be involved or related to a pathology. For example, oncogenes such as p53 are known to be involved or related to many forms of cancer and potentially to other pathologies (Sun, Y. et al. (1999) DNA sequence variants of p53: cancer and aging. Am J Hum Genet. 65(6):1779-82). The methods, compositions, and kits of the present disclosure can be designed to detect and/or quantify the amount of polynucleotides encoding an oncogene such as p53 or variant of the oncogene expressed in a host. The expression of specific polynucleotides in a host can then be used to assist in the diagnosis of the host. Exemplary oncogenes include but are not limited to growth factors, receptor tyrosine kinases, membrane associated non-receptor tyrosine kinases, G-protein coupled receptors, membrane associated G-proteins, serine/threonine kinases, and nuclear DNA-binding/transcription factors.

Exemplary growth factor oncogenes include the c-Sis gene (the v-sis gene is the oncogene in simian sarcoma virus) encodes the PDGF B chain. The v-sis gene was the first oncogene to be identified as having homology to a known cellular gene. The int-2 gene (named for the fact that it is a common site of integration of mouse mammary tumor virus) encodes an FGF-related growth factor. The KGF (also called Hst) gene also encodes an FGF-related growth factor and was identified in gastric carcinoma and Kaposi's sarcoma cells.

Receptor tyrosine kinases include c-Fms (fims)gene which encodes the colony stimulating factor-1 (CSF-1) receptor and was first identified as a retroviral oncogene. The Flg (flag) gene (named because it has homology to the Fms [fims] gene, hence fms-like gene) encodes a form of the FGF receptor. The Neu (new) gene was identified as an EGF receptor-related gene in an ethylnitrosourea-induced neuroblastoma. The conversion of proto-oncogenic to oncogenic Neu requires only a single amino acid change in the transmembrane domain. The Trk (track) genes encodes the NGF receptor-like proteins. The first Trk gene was found in a pancreatic cancer. Subsequently, two additional Trk-related genes were identified. These three are now identified as TrkA, TrkB and TrkC. The Met gene encodes the hepatocyte growth factor(HGF)/scatter factor (SF) receptor. The c-Kit gene encodes the mast cell growth factor receptor.

Membrane associated non-receptor tyrosine kinases include the v-src gene which was the first identified oncogene. The c-Src gene is the archetypal protein tyrosine kinase. The Lck gene

was isolated from a T cell tumor line (LYSTRA cell kinase) and has been shown to be associated with the CD4 and CD8 antigens of T cells.

G-protein coupled receptors include the Mas gene which was identified in a mammary carcinoma and has been shown to be the angiotensin receptor.

Membrane associated G-proteins include three different homologs of the c-Ras gene, each of which was identified in a different type of tumor cell. The Ras gene is one of the most frequently disrupted genes in colorectal carcinomas.

Serine/threonine kinases include the Raf gene which is involved in the signaling pathway of most RTKs. It is likely responsible for threonine phosphorylation of MAP kinase following receptor activation.

Nuclear DNA-binding/transcription factors include the Myc gene which was originally identified in the avian myelocytomatosis virus. A disrupted human c-Myc gene has been found to be involved in numerous hematopoietic neoplasias. Disruption of c-Myc has been shown to be the result of retroviral integration and transduction as well as chromosomal rearrangements. The Fos gene was identified in the feline osteosarcoma virus. The protein interacts with a second proto-oncogenic protein, Jun to form a transcriptional regulatory complex. The p53 gene was originally identified as a major nuclear antigen in transformed cells. The p53 gene is the single most identified mutant protein in human tumors. Mutant forms of the p53 protein interfere with cell growth suppressor effects of wild-type p53 indicating that the p53 gene product is actually a tumor suppressor.

Exemplary diseases and the polynucleotides involved in them that can be detected or quantified with the disclosed compositions and methods include but are not limited to the following: Li-Fraumeni Syndrome, Familial Retinoblastoma, Wilms Tumor, Neurofibromatosis Type 1, Neurofibromatosis Type 2, Familial Adenomatous Polyposis, Familial Breast Cancer (BRCA1 or BRCA 2), Multiple Endocrine Neoplasia Type 1, and Hereditary prostate cancer. Exemplary polynucleotides that can be detected or quantified according to the present disclosure include, but are not limited to, P53, RB1 WT1 NF1 NF2, APC, TSC1, TSC2, DPC4, Smad4, DCC, BRCA1, BRCA2 (SEQ. ID. No.: 8), STK11, HNPCC1, MSH2, MLH1, VHL, CDKN2A, PTCH, MEN1, RET, MEN2, p57, KIP2 MET, PTEN, HPC1, PRCA1, ATM, BLM, XPA, XPC,

XPD, XPE, XPF, FANCA, FANCC, FANCD, and FANCE. The nucleotide sequences of each of these genes is known in the art and available for example from GENBANK, and are incorporated by reference herein in their entirety.

Definitions

As used herein, "nucleic acid" or "nucleotide" can be a deoxyribonucleic acid, a ribonucleic acid, or a copolymer of deoxyribonucleic acid and ribonucleic acid. The sample of nucleic acids can be natural or synthetic. The sample of nucleic acid can be naturally occurring nucleic acid, and can be obtained from any organism. Some examples of organisms to which the method of the present invention is applicable include plants, microorganisms, viruses, birds, vertebrates, invertebrates, mammals, human beings, horses, dogs, cows, cats, pigs, or sheep. The target nucleic acid can occur naturally, or can be synthesized enzymatically in vivo, synthesized enzymatically in vitro, or synthesized non-enzymatically.

The sample containing the nucleic acid or polynucleotide of interest can comprise genomic DNA from an organism, RNA transcripts thereof, cDNA prepared from RNA transcripts thereof, or combinations of DNA and RNA. The sample containing the nucleic acid or acids of interest can also comprise extragenomic or episomal DNA from an organism, RNA transcripts thereof, or cDNA prepared from RNA transcripts thereof. Also, the nucleic acid or acids of interest can be synthesized by the polymerase chain reaction.

The nucleic acid of interest can comprise non-natural nucleotide analogs such as deoxyinosine or 7-deaza-2-deoxyguanosine. These analogues destabilize DNA duplexes and could allow a primer annealing and extension reaction to occur in a double-stranded sample without completely separating the strands.

The nucleic acid of interest can comprise one or more moieties that permit affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer. For example, the nucleic acid of interest can comprise biotin which permits affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer via binding of the biotin to the avidin family of molecules, which is attached to a solid support. The sequence of the nucleic acid of interest can comprise a DNA or RNA sequence that permits affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer via base pairing to

a complementary sequence present in a nucleic acid attached to a solid support. The nucleic acid of interest can be labeled with a detectable marker; this detectable marker can be different from any detectable marker present in the reagent or attached to the primer.

In this regard, the term "normal nucleotide" or "normal base" is defined as the wild-type or previously known standard nucleotide base from which a mutation is sought to be identified at the base site. By "standard nucleotide base", it includes any known base, which may include wild-type or a known mutant base so long as the base is known and it is desired to know its variant. Thus, as an example, normal base can be a known wild-type base for which a mutation is sought at the position. Alternatively, the known base can be a known mutant for which the presence of a wild-type base is sought at the position. Alternatively, the known normal base can be a known mutant for which another mutant variant base is sought. Therefore, the method of the invention can be applied to any known sequence that can be used to determine the presence of any other base variant at the site.

As used herein, the term "primer" or "oligonucleotide primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when placed under conditions that allow for synthesis of a primer extension product which is complementary to a nucleic acid (template) strand, in the presence of various factors such as for example, nucleotides and enzymes such as DNA polymerase, and at a suitable temperature and pH.

The term "primer" is alternatively defined as any nucleic acid fragment obtained from any source. For example, the primer can be produced by fragmenting larger nucleic acid fragments such as genomic DNA, cDNA or DNA that has been obtained through PCR. In other words, the nature of the primer is not limited by how the primer is obtained, whether it be by fragmenting naturally or synthetically occurring nucleic acid or by synthesizing the nucleic acid primer.

Furthermore, the primer can be oligodeoxyribonucleotide, a copolymer of oligodeoxyribonucleotides- , an oligoribonucleotides, a copolymer of ribonucleotides, or a copolymer of deoxyribonucleotides and ribonucleotides. The primer can be either natural or synthetic. The oligonucleotide primer can be synthesized either enzymatically in vivo, enzymatically in vitro, or non-enzymatically in vitro. The primer can be labeled with a detectable

marker; this detectable marker can be different from any detectable marker present in the reagent or attached to the nucleic acid of interest. In addition, the primer must possess sequence corresponding to the flanking sequence at a specific position of interest adjacent to, and upstream of, the nucleotide base to be identified.

In addition, the primer can be capable of hybridizing or annealing with nucleotides present in the nucleic acid of interest. One way to accomplish the desired hybridization is to have the template-dependent primer be substantially complementary or fully complementary to the known base sequence.

The oligonucleotide primer can comprise one or more moieties that link the primer to a solid support for affinity separation of the primer from the unincorporated reagent and/or the nucleic acid of interest. Such affinity moeties include, but are not limited to, digitonin, magnetic beads, and ligands, such as protein ligands, including antibodies. Preferably, the moiety is biotin. In the case of using biotin, the primer comprising biotin permits affinity separation of the primer from the unincorporated reagent and/or nucleic acid of interest via binding of the biotin to streptavidin which is attached to a solid support. The sequence of the oligonucleotide primer can comprise a DNA sequence that permits affinity separation of the primer from the unincorporated reagent and/or the nucleic acid of interest via base pairing to a complementary sequence present in a nucleic acid attached to a solid support.

As used herein, the term "primer extension reaction" refers to the reaction conditions in which the template-dependent nucleic acid synthesis reaction is carried out. The conditions for the occurrence of the template-dependent, primer extension reaction can be created, in part, by the presence of a suitable template-dependent enzyme. Some of the suitable template-dependent enzymes are DNA polymerases. The DNA polymerase can be of several types. The DNA polymerase must, however, be primer and template dependent. For example, E. coli DNA polymerase I or the "Klenow fragment" thereof, T4 DNA polymerase, T7 DNA polymerase ("Sequenase"), T. aquaticus DNA polymerase, or a retroviral reverse transcriptase can be used. RNA polymerases such as T3 or T7 RNA polymerase could also be used in some protocols. Depending upon the polymerase, different conditions must be used, and different temperature ranges may be required for the hybridization and extension reactions.

As used herein, the term "primer extension strand" includes the strand that is formed opposite the template in a duplex after the primer has been added. Preferably, the extension of the primer is terminated by the incorporation of the terminator to the primer extension strand.

As used herein, the term "template" is defined as a nucleic acid, including double strand DNA, single strand DNA and RNA, or any modification thereof, and can be any length or sequence.

As used herein, the term "terminator" or "chain terminator" is meant to refer to a nucleic acid base, such as A, G, C, T or U, or an analogue that effectively terminates the primer extension reaction when it is incorporated into the primer extension strand opposite the template strand. Preferably, the terminator is a dideoxynucleotide. Also preferably, the terminator is either unlabeled or is labeled so that it is distinguished from the label on the non-terminator. Also as used herein, when the term "terminator" or "chain terminator" are referred to in the singular, it does not mean that a single nucleotide molecule is used. Rather, the singular form of the term "terminator" refers to the type of nucleotide, nucleic acid base or nucleic acid analogue that is used in the assay. For example, if the terminator is ddA, then all of the ddA's in the aggregate are referred to in the singular form, and not just a single molecule of ddA. Alternatively, the "terminator" may be the absence of the specific type of nucleotide so that primer extension is stopped by the lack of the specific nucleotide at the locus. For example, if it is desired that the primer extension reaction be stopped opposite a "C" on the template strand, the non-terminating bases A, T and G should be included in the primer extension reaction mixture, but not "G", which is the complement of "C". Thus, the absence of the complementary base will cause termination of the primer extension reaction with a similar result as adding a dideoxy terminator nucleotide, for example.

As used herein, the term "non-terminator" or "non-chain terminator" includes a nucleotide base that does not terminate the extension reaction when it is incorporated into the primer extension strand. Preferably, at least one non-terminator in the primer extension reaction is labeled. Also as used herein, when the term "non-terminator" or "non-chain terminator" are referred to in the singular, it does not mean that a single nucleotide molecule is used. Rather, the singular form of the term "non-terminator" refers to the type of nucleotide, nucleic acid base or

nucleic acid analogue that is used in the assay. For example, if the terminator is G, then all of the G's in the aggregate are referred to in the singular form, and not just a single molecule of G.

As used herein, the term "mutant" or "mutation" indicates any base on the template strand that is different from the wild-type or normal base. The mutation that can be detected using the method of the instant invention can be any type of mutation at all, including, single base mutation, insertion, deletion, or gene translocation, so long as the base on the template directly opposite to the base immediately 3' to the annealed primer is affected.

As used herein, the term "label" refers to any molecule that is linked to the terminator or non-terminator nucleotide to provide a detectable signal. The label may be radioactive, chemiluminescent, protein ligand such as an antibody, or if a fluorescent group is used, a different fluorescent group may be used for each type of non-terminating nucleotide base. These fluorescent tags would have the property of having spectroscopically distinguishable emission spectra.

Alternatively, the method of determining the level of incorporation of a nucleotide base in the primer extension product can be measured by mass spectrometry techniques as exemplified in U.S. Pat. No. 5,885,775, which is incorporated herein by reference in its entirety.

As used herein, the phrase "high stringency hybridization conditions" refers to nucleic hybridization conditions, such as but not limited to a wash condition of 0.1.times.SSC, at 42.degree. C. Hybridization conditions generally can be found in general Molecular Biology protocol books, such as Ausubel et al., Current Protocols in Molecular Biology Greene and Wiley, pub. (1994), which is incorporated herein by reference in its entirety.

As used herein, "thin layer chromatography (TLC)" can be carried out in paper medium based on cellulose products, but can be made of any substance that allows for molecules to be finely divided and formed into a uniform layer. This substance includes, but is not limited to, inorganic substances such as silica gel, aluminum oxide, diatomaceous earth or magnesium silicate. Organic substances include, but are not limited to, cellulose, polyamide, or polyethylene powder. Thin layer chromatography methods are described generally in Chemical protocol books, such as generally set forth in Freifelder, Physical Biochemistry--Applications to Biochemistry and Molecular Biology, second ed., published by Freeman and Co. (1982), which

is incorporated herein by reference in its entirety, especially Chapter 8, which discusses chromatographic techniques, and in particular thin layer chromatography at pages 229-232.

A modification of the method for identifying and/or quantifying a nucleic acid of interest is to separate the primer extended strand from the nucleic acid of interest after the extension reaction by using appropriate denaturing conditions. The denaturing conditions can comprise heat, alkali, formamide, urea, glyoxal, enzymes, and combinations thereof. The denaturing conditions can also comprise treatment with 2.0N NaOH.

It can be appreciated by a person of skill in the art that the terminator can be labeled with a different label from the non-terminator, which can then be used to differentiate between incorporation of terminator or non-terminator in the primer extension strand. The terminator exemplified as being the absence of the particular type of nucleotide in the present application only for purposes of simplicity of illustration, but this illustration should not be construed to limit the claims in any way. Differentially labeled or unlabeled terminator is also encompassed by the invention, so long as the label on the terminator is different from the label on the non-terminator.

It can also be appreciated by a person of skill in the art that so long as the sequence of the template is at least partially known, a primer can be designed that binds to the template strand such that the binding of the primer on the template strand can occur. It can also be appreciated by a person of skill in the art that the method of the invention can be practiced by using several primers in one or more assay tube.

A feature of the method of the invention is that strong signal can be generated if the non-terminators are uniformly labeled because of the additive signal effect achieved by the incorporation of several labeled non-terminators incorporated in the primer extension strand. Accuracy is enhanced when signals are observed from using different labels specific to various terminators or non-terminators.

It is also an object of this invention to provide a kit and reagents for rapidly and accurately determining the presence or absence of a target nucleic acid in a sample quantitatively or non-quantitatively as desired. Each component of the kit(s) may be individually packaged in its own suitable container. The individual containers may also be labelled in a manner which identifies the contents. Moreover, the individually packaged components may be placed in a

larger container capable of holding all desired components. Associated with the kit may be instructions which explain how to use the kit. These instructions may be written on or attached to the kit.

The following examples are offered by way of illustration of exemplary embodiments of present disclosure, and not by way of limitation.

EXAMPLES

Example 1

Total RNA was extracted from rat brain by RNAzol B (Tels-tel, TX) method. The concentration of total RNA was measured by O.D. 260 nm absorbance. Total RNA was diluted by RNase-free diethylpyrocarbonate (DEPC) treated water. 5 μl of diluted RNA solution with different amounts as indicated in Table 2 was aliquoted into each tube and then mixed with 1 μl synthetic oligonucleotide primer 5'-GTGGGAACCGTGTCA-3' (SEQ ID NO:1), which is a sequence matched to a rat brain specific cDNA (unpublished data). The RNA-primer mixture was heated at 70°C for 3 minutes and incubated on ice for 3 minutes. After quick spinning the tube, the primer extension reaction was started by adding 14 μl reaction mix containing Tris-HCl buffer (pH 7.5) at final concentration of 20 mM, 15 units RNase inhibitor, 0.5 mM dATP, dGTP, 1 μl dCTPα^{32}P and 10 units MMVL-reverse transcriptase. The reaction was performed at 37°C for 20 minutes and stopped by heating the reaction tube at 100°C for 2 minutes. A 1 μl reaction mixture was applied to a thin layer chromatography (TRIM USA, Maryland) to separate out free dCTPα^{32}P. The radioactivity of the labeled primer was then subjected to counting by scintillation counter (Beckman LS 5000). The results are shown in Table 2.

Table 1 provides a comparison of the various factors associated with Northern blotting, RNase protection assay and an exemplary embodiment of the multiple primer extension method of the present disclosure.

Table 1

Comparison of MPE method with Northern analysis and RNase protection assay

Biohazard/Radioisotope

Methods	Time consuming	Experiment procedure	Sensitivity Principle		Expense	Wastes
Northern analysis	2-3 days	Running RNA gel RNA Transfer Prepare probe Hybridization	5 μg	Hybridization only	High	High
Rnase Protection Assay	2-3 days	Prepare template DNA Prepare RNA probe Hybridization Enzyme digestion Running gel	1μg	Hybridization and enzyme digestion	High	High
MPE	1 hour	Primer extension	1μg	Hybridization and specific extension	Low	Low

Table 2

RNA amount (ng)	Labeled primer (epm)
20	31552
10	29756
5	26066
2	16779
1	11156
0.5	6587
0	6703

Example 2

Total RNA from brain was purchased from Ambion (TX, USA). The original RNA was diluted to a concentration showed in Table 3 below with nuclease free water. Two primers for detecting human beta actin RNA were synthesized. The sequence of primer 1 was 5'-TCCACGTCACACTTC (SEQ. ID. NO.: 6), and sequence for primer 2 is 5'-CACCACGGCCGAGCGGGAAA (SEQ. ID. NO.: 7). The 5' end of primer 2 was covalently

attached to a 96-well microplate. Human beta actin gene expression was assayed by adding 5 μM of Primer 1, 1.5-12 ng RNA, to a final volume of 10 μl. This primer-RNA mixture was then heated at 70°C for 3 minutes and then incubated on ice. A reaction mixture was add to the primer-RNA mixture to make a reverse transcription reaction mix with final concentration of 1mM of dCTP, dGTP, dTTP; 100 units of Reverse transcriptase, 1x reverse Transcriptase buffer, and 10 units of RNase inhibitor (Ambion, TX USA). The isometric primer extension was performed by incubation of the reaction mixture at 37°C for 15 minutes and then 95 °C for 5 minutes. After the reverse transcription reaction, 5 μl of reaction product was transferred to each well of microtiter plate coated with Primer 2. A 25 μl aliquot of a second reaction mix was added to each well to make the final concentration of 1mM of dCTP, dGTP, dTTP, dATP, 2 mM biotin labeled dATP, 10 units of Taq polymerase, 1x Taq polymerase buffer. The second round of isometric primer extension reaction was performed with following temperature cycles:

30 cycles	95 °C 1 minute
	60 °C 1 minute
	72 °C 1 minute
1 cycle	95 °C 5 minutes

After the second extension reaction, the wells were washed three times. 50 μl of horseradish peroxidase conjugated streptavidin (Pierce, IL USA) was added to each well and plates were incubated at 25°C for 30 minutes. To develop the color, the wells were washed three times, and 50μl of horseradish peroxidase substrate ABTS (2,2'-AZINO-bis [3-ethylbenziazoline-6-sulfonic acid) (Moss, Inc. MD USA) was added to each well. The plates were incubated at 25°C for 30 minutes and absorbance at 405 nm was determined using a microplate reader (1420 VICTOR 2 multilabel counter, WALLAC USA). The results are shown in Table 3 and Figure 5.

The test results show a correlation between the amount of target RNA and the absorbance at 405 nm.

Table 3

Total RNA Concentration	OD 405 nm	
	Test 1	Test 2
1.5 ng	0.303	0.352
3 ng	0.685	0.757
6 ng	1.244	1.09
12 ng	1.684	1.858

Example 3

The expression level of two cancer related genes in cancer tissue was analyzed using the disclosed method. The detection primers were designed to bind to the target sequence of B-raf and P53 genes. For B-raf, the first primer sequence is 5'-AACGATAGGTTTTTGTGGGTGA (SEQ. ID. NO.: 9) and second primer is 5'-CACGGAGCAACCCCAAG (SEQ. ID. NO.: 10). For P53 gene, the sequence of first primer is 5'-GGGTGAAATATTCTCCATCCA (SEQ. ID. NO.: 11) and the second primer is 5'-CCCCAGCCAAAGAAGAAACCAC (SEQ. ID.NO.12). The second primers were attached to a 96 well microplate.

Total RNA was obtained from human breast cancer tissue and 200 ng total RNA was used for the test to detect the expression level of B-raf and P53 genes. The results are showed in Figure 6. In this example, a sample can be screened for the presence or absence of a polynucleotide having at least a portion of the known B-raf or P53 sequence or a known variation thereof. The amount of B-raf or P53 polynucleotide or variation thereof detected in the sample can then be used to diagnose the host or assess the risk of pathology development in the host.

All of the above steps involve chemistries, manipulations, and protocols that have been, or are amenable to being, automated. Thereby, incorporation of the preferred mode of practice of this invention into the operation of a suitably programmed robotic workstation should result in significant cost savings and increases in productivity for virtually any diagnostic procedure that depends on the detection of specific nucleotide sequences or sequence differences in nucleic acids derived from biological samples.

Many obvious variants are possible within the realm of the present invention. For example, not just one type of nucleotide but two or three types of nucleotides maybe absent in the primer extension reaction. Also, various labels could be used, which are not limited to radioactive nucleotides but can be fluorescent, as well as enzymatic.

All of the references cited herein are incorporated by reference in their entirety.